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# Development of bioreactors for application of biocatalysts in biotransformations and bioremediation*

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*Abstract*: Biotransformation systems, whether used for environmentally benign biocatalysis of synthetic reactions, or bioremediation of pollutants, require suitable biocatalysts and suitable bioreactor systems with particular characteristics. Our research focuses on the bioconversion of organic compounds, many of which are industrial residues, such as phenols, polyaromatic hydrocarbons, heterocyclic compounds, and polychlorinated biphenyls. The purpose of such biotransformations can be twofold: firstly, to remove them from effluents and convert them to less toxic forms, and secondly, to convert them into products with economic value. We conduct research in utilizing various isolated-enzyme and whole-cell biological agents; bioreactors, including novel membrane bioreactors, are used as a means of supporting/immobilizing, and hence applying, these biocatalysts in continuous systems. In addition, the enzyme systems are characterized biochemically, to provide information which is required in modification, adaptation, and scale-up of the bioreactors. The paper summarizes research on application of biofilms of fungal and bacterial cells and their enzymes, including hydrolases, polyphenol oxidase, peroxidase and laccase, in bioreactor systems including continuously operating membrane bioreactors.

## INTRODUCTION

Biotransformations involve the use of biological agents, in the form of whole cells or isolated enzymes, to catalyze chemical reactions. Such biotransformations systems may be used for environmentally benign biocatalysis of synthetic reactions, bioremediation of pollutants, or waste beneficiation, a combination of these in which the biological agents convert industrial residues to useful chemical products. In each case, suitable biocatalysts, and suitable bioreactor systems, each with particular characteristics, are required. Our research focuses on the bioconversion of organic compounds, many of which are industrial residues, such as phenols, polyaromatic hydrocarbons, heterocyclic compounds, and polychlorinated biphenyls. The purpose of these biotransformations is firstly, to remove them from effluents and convert them to less toxic products, and secondly, to convert them into products with economic value. These reactions can be accomplished utilizing various isolated-enzyme and whole-cell biological agents.

A critical consideration in any biotransformation application is the acquisition and development of a suitable biological catalyst. This may require application of various microbiological, biochemical engineering and, particularly, molecular, technologies which are now accessible. Thus, exploration of biodiversity and/or molecular engineering are important steps in the acquisition of novel enzyme activities. The majority of enzymes currently being applied or developed for biotransformations are micro-

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bial in origin, and the field of molecular genetics has provided much scope for the isolation and manipulation of enzymes to produce biocatalysts with predetermined properties.

In most biotransformation processes, immobilization or stabilization of the biological agent confers significant advantages, such as facilitating reuse of an enzyme, containment of cells, or support of a growing biofilm for use in a continuous system. Bioreactors, including novel membrane bioreactors are used as a means of supporting or immobilizing, and hence applying, the biocatalysts in continuous systems. In addition, the biotransformation systems can be characterized biochemically, to provide information which can then be used in mathematically modeling the biotransformations, facilitating rational approaches to modification, adaptation, and scale-up of the bioreactors.

## PRODUCTION OF BIOCATALYSTS

Biocatalyst development requires, initially, identification of the required enzyme activity, in terms of the desired chemical reaction substrates and products, and thereafter, the identification of sources of such enzyme activity. Conventional microbiological approaches using selective screening may be adequate for this purpose, but the range of sophisticated modern molecular technologies now available has provided the biotransformations chemist with an immensely enhanced choice of potential enzyme sources. Exploration of extreme environments, and exploitation of genomes using advanced technologies such as total environmental DNA sampling, cloning technologies, and genetic manipulation, have altered the approach in biocatalysis to the point where rather than develop a purpose for an enzyme, we design and develop an enzyme for a purpose.

Once a suitable source of the desired enzyme activity has been found, an efficient production system for the enzyme would be required, whether it is to be applied as an isolated enzyme or encapsulated in cells. In a typical conventional biocatalysis process, the cells would be grown in a fermentation system, then harvested and used as whole cells, or the enzymes would be extracted and then used in an isolated form, for the biocatalytic reaction. The fermentation would be optimized to produce maximum biomass, and the cells would be harvested when the desired enzyme activity was maximal.

Further biocatalyst development would involve characterization of the enzyme production by the cells and the kinetics and substrate selectivity of the enzymes involved. Optimization of fermentation and biotransformation reaction conditions may involve extensive investigation and modification of the biocatalyst production system and the reactor conditions. For industrial application, further development would require stabilization of the biocatalyst so as to ensure consistent biocatalytic activity over prolonged reaction times, in terms of product fidelity as well as chemical productivity and biological stability.

The ideal biotransformation system would include:

- an efficient enzyme production system
  - -using a readily culturable microbial source
  - -genetically stable, nonpathogenic strain
- an efficient biocatalyst
  - -used as resting cells or purified enzymes
  - -highly stereoselective
  - -high activity in the desired reaction
  - -flexible substrate selectivity
  - -minimal side reactions
- a stable biocatalyst
  - -stable under optimal reaction conditions
  - -menable to immobilization/stabilization
  - -amenable to bioreactor constraints

We have a number of choices, in terms of improving biocatalysts for industrial application, including metabolic engineering of the biological source (the cells), for instance, to alter the enzyme

expression by the cells. Alternatively, genetic engineering might lead to, for instance, enhanced enzyme selectivity or elimination of side reactions, or production of recombinant organisms in which desirable characteristics of different enzymes from more than one organism can be combined.

An alternative to molecular approaches is the reaction engineering approach, in which the bioreactor conditions are manipulated to provide an environment suited to the biocatalyst and the biotransformation.

Reaction engineering allows for the development of:

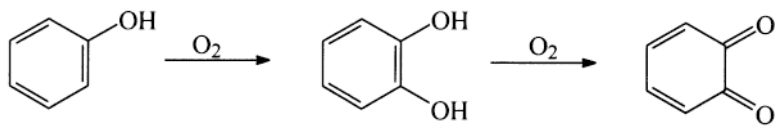
- continuous fermentation/biotransformation processes
- continuous addition of substrate and recovery of product
- efficient use of immobilized or stabilized enzymes/cells
- use of heterogeneous solvent systems (organic/aqueous)
- expanded substrate range

While the scope for development of biotransformation systems is very broad, any particular reaction system would possess specific characteristics and would require reactor development to be customized at least to some extent. The following sections describe some specific biotransformation systems which have been successfully developed, and which illustrate the considerations described above.

## OXIDATIVE ENZYME-CATALYZED REACTIONS

### Polyphenol oxidase biotransformations

Polyphenol oxidase (PPO) catalyzes the *ortho*-hydroxylation of phenols to catechols, and these are then further oxidized by the enzyme to form *ortho*-quinones (Scheme 1). The reaction uses molecular oxygen and requires no additional cofactors. Catechols are valuable chemical synthons not readily prepared by conventional methods, and the quinone products can be reduced to yield catechols, or polymerized or adsorbed to remove them from the system [1].




**Scheme 1** Polyphenol oxidase-catalyzed reaction.

The PPO enzyme system has been investigated extensively for bioremediation, but less attention has been given to its application in synthesis, largely because of its characteristic product inhibition which is due to *ortho*-quinone products interacting with the active site of the enzyme [2]. In attempts to minimize this effect, we have conducted investigations of membrane immobilization systems and their effect on the nature of the product mixture obtained from the bioconversion of phenols in synthetic effluents. Capillary-membrane immobilization of the enzyme in a customized bioreactor can increase the stability of the enzyme by allowing separation of the product. Furthermore, integration of a viscous chitosan adsorbent in the capillary bioreactors has been shown to be even more effective in removing quinones and protecting the enzyme from product inhibition [3].

In addition, immobilization of PPO on certain membrane supports and under optimal conditions can prolong the enzyme activity and facilitate the production of catechol products, and minimizing quinone formation and obviating the need for reduction of the quinones [4]. Mathematical modeling of the reaction system allows us to identify the reaction conditions, and times during the reaction, at which catechol production is maximal, i.e., under which catechol could be isolated rather than allowing it to be further converted to quinone (Fig. 1). Such modeling also provides a means of formulating reaction

rate constants under nonconventional conditions where classical enzyme kinetics approaches cannot be applied (Fig. 2).

In cases where the phenol substrates or products of the PPO reaction are hydrophobic, organic medium systems can be utilized, in which case the enzyme can be immobilized (for instance, on glass) and hydrated under controlled conditions which can be enhanced by the addition of small amounts of surfactant. Thus, even sterically demanding substrates such as 4-*tert*-butylphenol can be converted by PPO [5].

## Peroxidase-cataylzed biotransformations

Peroxidases catalyze oxidations utilizing hydrogen peroxide, and generally producing free radicals which can subsequently be coupled or reacted further. Peroxidases are produced by many plant and fungal sources, often, in the latter case, as extracellular cell products which can be produced continuously in whole-cell bioreactors. The merits of utilization of these enzymes include properties such as low specificity (and hence broad substrate range) and the fact that the reactions result in chemical activation of relatively unreactive substrates to produce more active synthons. Thus, for example, carbon–carbon bonds or carbon–hetero bonds can be formed (Scheme 2) or polymeric structures such as lignins, tannins, and polyphenolics can be broken down to yield monomers, depending on the particular enzyme and conditions used.

## Laccase biotransformations

Laccases are also rather nonspecific in their reactions, which include radical-mediated carbon–carbon bond cleavage, aromatic odixations, and coupling reactions (Scheme 3). We have established a method

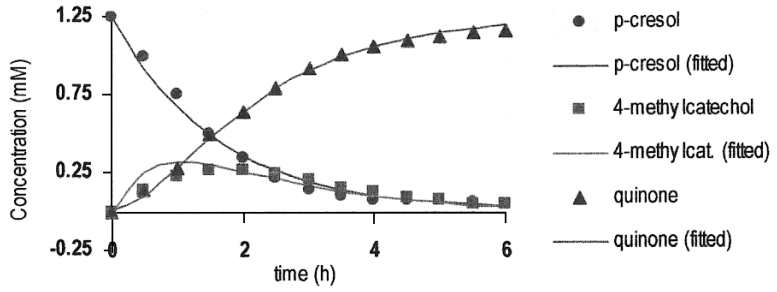



**Fig. 1** The modeled polyphenol oxidase-catalyzed reaction of *p*-cresol, showing catechol concentration during reaction.

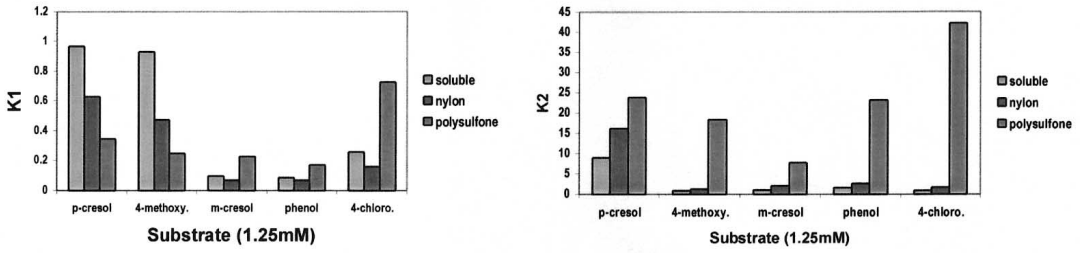
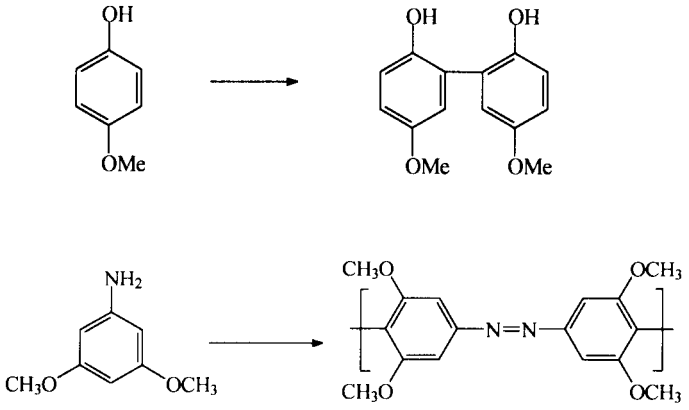
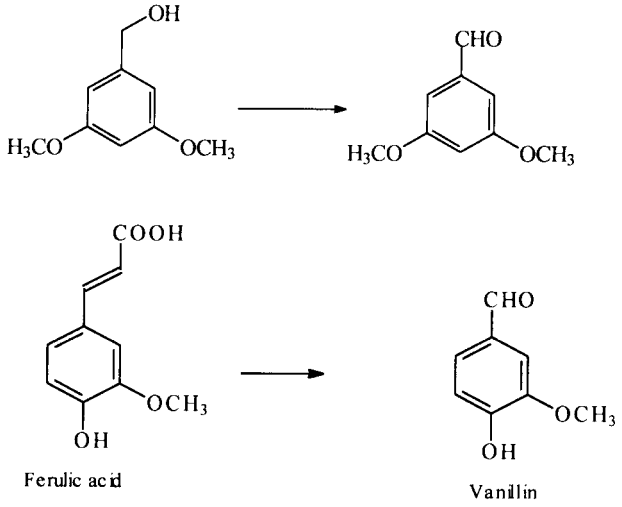



**Fig. 2** Examples of reaction rate constants obtained from modeling of reactions using polyphenol oxidase immobilized on various membranes.

of producing laccase in a continuously operating bioreactor (Fig. 3), and have tested the capacity of the systems to transform phenols and lignins as model substrates. In a study of bioremediation of phenols, a fungal biofilm system in a capillary membrane bioreactor has been operated continuously for 4 months, and the productivity in terms of bioconversion of cresol was demonstrated to be 1.4 g per day per $m^2$, or 0.8 mg per day per g biomass (Table 1) [6].




**Scheme 2** Peroxidase-catalyzed reactions.




**Scheme 3** Laccase-catalyzed reactions.

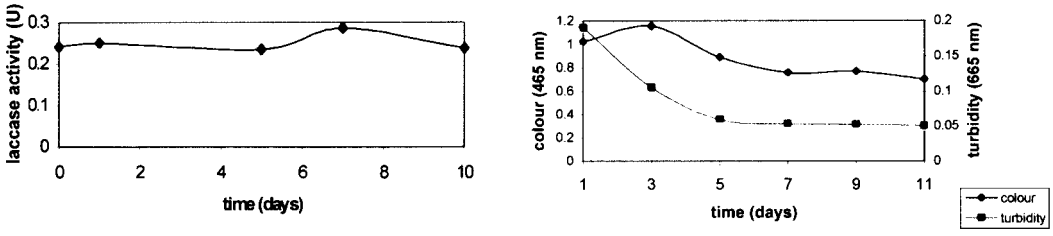



**Fig. 3** Continuous production of fungal laccase in an uplift reactor, and application in decolorization of industrial effluent.

## Bacterial systems

The work described above focuses mainly on enzymes obtained from fungal sources, but the technology is also presently being adapted for the immobilization of bacteria which produce similar oxidative enzyme systems. The research has involved the development of techniques for growth to steady state of biofilms of the fungi whose enzymes then utilized *in situ* in whole-cell bioreactors. Alternatively, the enzymes can be isolated and used in immobilized-enzyme membrane bioreactors, for biotransformation reactions. New directions include developing molecular and biochemical engineering techniques for improving the applicability of these enzymes.

The bioreactor technology described above can be adapted for the immobilization of biofilms of any number of different microorganisms. A very large number of biotransformations processes use hydrolytic reactions, including lipases, proteases, esterases, nitrilases, etc. An interesting group of hydrolases, the hydantoinases, can be used to produce chiral amino acids and their derivatives, as chiral starting materials in the production of pharmaceuticals such as antibiotics. The reactions are stereoselective, giving D- or L-products [7]. These compounds can be efficiently synthesized by the biocatalytic conversion of substituted hydantoins, in reactions catalyzed by microbial isolated enzymes or resting cells, in solution or immobilized in bioreactors.

**Table 1** Comparison of phenol conversion capacity of fungal oxidase in shaken, static, and membrane-supported cultures.

| | | Mass of substrate converted per mass biomass (mg/g/d)[1] | | |
|---|---|---|---|---|
| Model pollutant | Initial concentration | Shaken cultures[2] | Static cultures[2] | Membrane bioreactor[3] |
| p-Cresol | 0.5 | 0.27 | 0.36 | - |
| | 1.5 | 0.92 | 1.07 | 0.39 |
| | 2.5 | 1.23 | 1.73 | 0.97 |
| | 5.0 | 2.37 | 2.61 | 1.79 |
| Phenol | 0.5 | 0.39 | 0.16 | - |
| | 1.5 | 0.42 | 0.47 | 0.31 |
| | 2.5 | 1.13 | 1.67 | 0.73 |
| | 5.0 | 3.38 | 3.35 | 1.35 |

[1] Average over 6 days during which phenolic substrate was present.
[2] Biomass present at introduction of substrate = 1.16 g/100mL.
[3] Biomass present at introduction of substrate = 20 g/bioreactor.

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